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# *Hansenula polymorpha Pex1p* and *Pex6p* are Peroxisome-associated AAA Proteins that Functionally and Physically Interact

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We have cloned the *Hansenula polymorpha PEX1* and *PEX6* genes by functional complementation of the corresponding peroxisome-deficient (*pex*) mutants. The gene products, HpPex1p and HpPex6p, are ATPases which both belong to the AAA protein family. Cells deleted for either gene (Δ*pex1* or Δ*pex6*) were characterized by the presence of small peroxisomal remnants which contained peroxisomal membrane proteins and minor amounts of matrix proteins. The bulk of the matrix proteins, however, resided in the cytosol. In cell fractionation studies HpPex1p and HpPex6p co-sedimented with the peroxisomal membrane protein HpPex3p in both wild-type cells and in Δ*pex4*, Δ*pex8* or Δ*pex14* cells. Both proteins are loosely membrane-bound and face the cytosol. Furthermore, HpPex1p and HpPex6p physically and functionally interact *in vivo*. Overexpression of *PEX6* resulted in defects in peroxisomal matrix protein import. By contrast, overexpression of *PEX1* was not detrimental to the cells. Interestingly, co-overproduction of HpPex1p rescued the protein import defect caused by HpPex6p overproduction. Overproduced HpPex1p and HpPex6p remained predominantly membrane-bound, but only partially co-localized with the peroxisomal membrane protein HpPex3p. Our data indicate that HpPex1p and HpPex6p function in a protein complex associated with the peroxisomal membrane and that overproduced, mislocalized HpPex6p prevents HpPex1p from reaching its site of activity. 

KEY WORDS — AAA protein family; methylotrophic yeast; microbody; *PEX* gene; peroxisome biogenesis

## INTRODUCTION

Microbodies (peroxisomes, glyoxysomes, glycosomes) are vital organelles, present in virtually all eukaryotic cells. Their metabolic function is highly diverse, dependent on the organism in which they occur. Examples of important microbody-bound metabolic pathways are photorespiration (plants), cholesterol metabolism (mammals), glycolysis (trypanosomes), penicillin biosynthesis (fungi) and primary metabolism of certain carbon or organic nitrogen sources (fungi, including yeasts) (Müller *et al.*, 1991; Veenhuis and Harder, 1991; Van den Bosch *et al.*, 1992; Reddy *et al.*, 1996).

Until recently, the generally accepted view on microbody biogenesis was that the organelles develop by fission from pre-existing ones; organellar growth was thought to be accomplished by

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post-translational import of matrix and membrane proteins by processes comparable to those observed in mitochondria and plastids (Lazarow and Fujiki, 1985). However, recent breakthroughs in microbody research brought about major changes in these views. These include the finding that peroxisomal matrix proteins may oligomerize prior to import and the observation that receptors which recognize peroxisomal targeting signals may shuttle between the cytosol and the peroxisome (reviewed by Erdmann *et al.*, 1997). In addition, studies on novel genes/proteins involved in peroxisome biogenesis (*PEX* genes/peroxins; Distel *et al.*, 1996) suggested that the endoplasmic reticulum (ER) and membrane vesicles may be involved in peroxisome biogenesis (Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998).

In the present paper we describe the isolation and characterization of *Hansenula polymorpha PEX1* and *PEX6*. Both genes encode members of the AAA protein family (**A**TPases **A**ssociated with various cellular **A**ctivities). AAA proteins share the presence of one or two AAA modules, characterized by a putative ATP binding site and a second region of homology with an unknown function (Confalonieri and Duguet, 1995). AAA proteins containing a single AAA module include bacterial ATP-dependent metalloproteases and subunits of the proteasome in eukaryotes (Rechsteiner *et al.*, 1993). Two AAA modules are found in AAA proteins implicated in vesicle fusion processes (e.g. *Saccharomyces cerevisiae* Sec18p and Cdc48p) or peroxisome biogenesis (Pex1p and Pex6p) (Confalonieri and Duguet, 1995). *PEX1* and *PEX6* genes have been cloned from *S. cerevisiae* (Erdmann *et al.*, 1991; Voorn-Brouwer *et al.*, 1993), *Pichia pastoris* (Spong and Subramani, 1993; Heyman *et al.*, 1994) and man (Yahraus *et al.*, 1996; Fukuda *et al.*, 1996; Reuber *et al.*, 1997; Portsteffen *et al.*, 1997; Tamura *et al.*, 1998a). For *Yarrowia lipolytica* (Nuttley *et al.*, 1994) and rat (Tsukamota *et al.*, 1995) only the *PEX6* gene has been described. Surprisingly, so far the subcellular location of Pex1p and Pex6p is still unclear. Also their specific function in peroxisome biogenesis remains speculative. In this contribution we provide evidence that, in the methylotrophic yeast *H. polymorpha*, Pex1p and Pex6p physically and functionally interact and form a complex that is loosely associated with the outer surface of the peroxisomal membrane.

## MATERIALS AND METHODS

### *Organisms, media and growth conditions*

The following strains were used in this study: *H. polymorpha* strain NCYC495 and auxotrophic derivatives thereof (Gleeson and Sudbery, 1988); *per4-152* (*leu1.1*); *per5-127* (*leu1.1*) (Titorenko *et al.*, 1993); Δ*pex4* (*leu1.1*) (Van der Klei *et al.*, 1998a); Δ*pex8* (*ura3*) (Waterham *et al.*, 1994); Δ*pex14* (*leu1.1*) (Komori *et al.*, 1997) and CBS4732 (CBS collection, The Netherlands); *S. cerevisiae* SFY526 and HF7c (ClonTech Laboratories, Inc); and *Escherichia coli* DH5α (Sambrook *et al.*, 1989). *H. polymorpha* strains were grown at 37°C in rich medium containing 1% yeast extract, 2% peptone and 1% glucose (YPD), selective minimal media containing 0·67% Yeast Nitrogen Base without amino acids (Difco), supplemented with 1% glucose (YND) or 0·5% methanol (YNM), or mineral medium supplemented with 0·5% carbon source and 0·25% nitrogen source (Van Dijken *et al.*, 1976). When required, amino acids and uracil were added to a final concentration of 30 μg/ml. *S. cerevisiae* strains were cultured as recommended by the supplier of the MATCHMAKER system (Clontech). *E. coli* DH5α was grown at 37°C in LB medium supplemented with the appropriate antibiotics.

### *DNA procedures*

*H. polymorpha* cells were transformed using the electroporation method (Faber *et al.*, 1994). Recombinant DNA manipulations were as described by Sambrook *et al.* (1989). Southern blot analysis was performed using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham) according to the manufacturer's instructions. Polymerase chain reaction-mediated DNA amplification was performed with *Pwo*-polymerase (Boehringer–Mannheim, Germany), according to the manufacturer's instructions.

### *Cloning and Sequence Analysis of* PEX1 *and* PEX6

To isolate the *PEX1* and *PEX6* genes, the *H. polymorpha* mutants *per4-152* and *per5-127* were electrotransformed with an *H. polymorpha* genomic DNA library constructed in vector pYT3 (Tan *et al.*, 1995). Leucine prototrophic transformants were screened for the ability to grow on methanol ($Mut^+$), and their plasmid content was rescued in *E. coli*. Retransformation of the

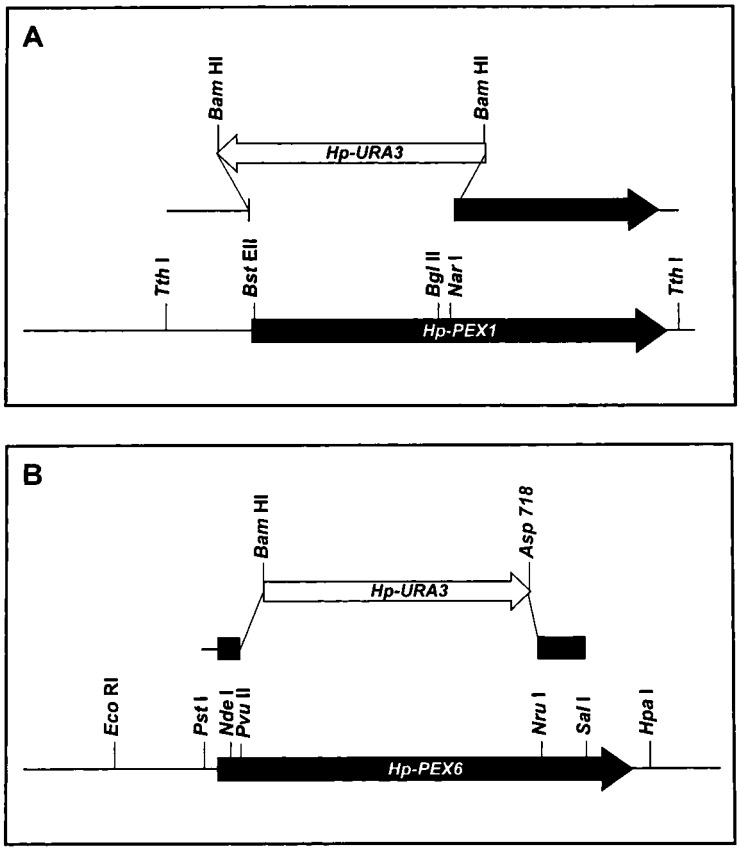



Figure 1. Schematic representation of the genomic regions comprising *H. polymorpha PEX1* (A) and *PEX6* (B) and the strategies used to construct deletion mutants for both genes. Only relevant restriction sites are indicated.

*per4-152* and *per5-127* strains with these plasmids again resulted in restoration of growth on methanol. For both genes the smallest complementing fragment was cloned in *Sma*I-digested pBluescript II $SK^+$ (Stratagene) and a series of nested deletions was generated by the limited exonuclease III digestion method (Sambrook *et al.*, 1989). Double-stranded DNA sequencing of the resulting subclones was carried out on an ABI 313A automatic sequencer (Applied Biosystems) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit. In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the DNA sequences. For analysis of the DNA sequences and deduced amino acid sequences, the PC-GENE program, release 6.70 (IntelliGenetic Inc., Mountain View, CA), was used. The TBLASTN algorithm (Altschul *et al.*, 1990) was used to screen databases at the National Center for Biotechnology Information (Bethesda, MD). The relevant nucleotide sequences have been deposited at GenBank and were assigned Accession Nos AF129873 (*PEX1*) and AF129874 (*PEX6*).

### *Construction of* PEX1 *and* PEX6 *deletion mutants*

To disrupt the wild-type *PEX1* gene, a disruption cassette was constructed by cloning a 2·3 kb *Bam*HI fragment (blunted by Klenow treatment) containing the *H. polymorpha URA3* gene (Merckelbach *et al.*, 1993) between the *Bst*EII and *Nar*I sites (both blunted) of the *per4-152* complementing insert in pBluescript (Figure 1A). The disruption cassette was isolated by *Tth*I digestion and used to transform *H. polymorpha* NCYC495 *leu1.1 ura3*. A similar disruption cassette was constructed to disrupt *PEX6*: the *H. polymorpha*

*URA3* gene was cloned between the *Pvu*II and *Nru*I sites of the *per5-127* complementing fragment in pBluescript. In this case the disruption cassette used to transform *H. polymorpha* was isolated with *Pst*I and *Sal*I (Figure 1B). Uracil-prototrophic transformants were selected and tested for the ability to grow on YNM plates. Mut$^-$ strains were examined for correctly targeted genomic integration by Southern blot analysis (data not shown). Segregation, complementation and linkage analyses of the deletion mutants were as described previously (Titorenko *et al.*, 1993), and demonstrated that the cloned *PEX1* and *PEX6* genes encode the authentic genes that are defective in *per4-152* and *per5-127*, repectively, and do not represent suppressors.

### *Construction of* PEX1 *and* PEX6 *overexpression strains*

To establish overexpression of *PEX1* and *PEX6*, the genes were cloned behind the strong regulatable alcohol oxidase promoter ($P_{AOX}$). To this purpose a *Bam*HI site was introduced upstream of the startcodon of *PEX1* and *PEX6* by PCR using either the PEX1–ATG primer (5′ TAC CGG ATC CTT ATG GAC TCA CAG GAG 3′) or the PEX6–ATG primer (5′ AGA GGA TCC ATG CCT GGT CTT GTG GAA GC 3′).

For *PEX1*, a *Bam*HI–*Bst*EII fragment originating from the *PEX1*-specific PCR product, and a *Bst*EII–*Eco*RV fragment from a subclone of the original *per4-152* complementing fragment, were cloned between the *Bam*HI and *Xba*I (blunted) sites of pBluescript II SK$^+$, yielding plasmid pBSK–PEX1ORF. From this plasmid, the *PEX1* open reading frame was isolated as a *Not*I (blunted)–*Bam*HI fragment and ligated between the *Sma*I and *Bam*HI sites of vector pHIPX4-B (Komori *et al.*, 1997), resulting in plasmid pHIPX4–PEX1. To enable overexpression of *PEX1* in a strain already overexpressing *PEX6*, we first constructed vector pHI1 by inserting a 1·8 kb *Bam*HI–*Asp*718 (both blunted) fragment containing the *H. polymorpha URA3* gene in the *Nde*I (blunted) site of pUC19 (Yanisch-Perron *et al.*, 1985). Subsequently, an *Eco*RV fragment of pHIPX4–PEX1 containing the $P_{AOX}$*PEX1* cassette was cloned in the *Sma*I site of pHI1, resulting in plasmid pHI1–$P_{AOX}$PEX1.

For *PEX6*, a *Bam*HI–*Nde*I fragment originating from the *PEX6*-specific PCR fragment and a *Nde*I–*Hpa*I fragment from the original *per5-127* complementing clone (Figure 1B) were cloned between the *Bam*HI and *Sma*I sites of plasmid pHIPX4–HNBESX (K.B. Rechinger, unpublished results), a derivative of pHIPX4 (Gietl *et al.*, 1994), resulting in plasmid pHIPX4–PEX6.

For targeted integration, plasmids pHIPX4–PEX1 and pHIPX4–PEX6 were linearized with *Stu*I in the $P_{AOX}$ region and used to transform the *H. polymorpha* strains NCYC495 (*leu1.1 ura3*), Δ*pex1*(*leu1.1*) and Δ*pex6* (*leu1.1*). Plasmids pHI1–$P_{AOX}$PEX1 and pHI1 (used as control) were linearized with *Spe*I in the *URA3* region to direct integration at that genomic site and used to transform *H. polymorpha* NCYC495::[$P_{AOX}$*PEX6*]$^{5x}$ (*ura3*). Selection on integration of the plasmids was performed as described by Faber *et al.* (1993). Southern blot analysis was used to detect single-copy or multi-copy integration at the *AOX* or *URA3* loci (data not shown).

### *Generation of α-HpPex1p and α-HpPex6p antibodies*

For the generation of antibodies against HpPex1p and HpPex6p, we constructed plasmids that allow synthesis in *E. coli* of fusion proteins between the maltose-binding protein and the N-terminus of either HpPex1p or HpPex6p, that were also 6xHis-tagged at their C-termini. First, a 6xHis cassette was constructed by annealing the primers 6HIS1 (5′ AAT TCG TCG ACA TCA CCA TCA CCA TCA CTA ATA GC 3′) and 6HIS2 (5′ CCG GGC TAT TAG TGA TGG TGA TGG TGA TGT CGA CG 3′) and cloning the fragment between the *Eco*RI and *Xma*I sites of pHIPX4–HNBESX, resulting in plasmid pHIPX4–HIS6. For *PEX1*, a 1·5 kb *Bam*HI–*Bgl*II (two-base fill-in) fragment from pHIPX4–PEX1, encoding the first 477 amino acids of HpPex1p, was cloned between the *Bam*HI and *Sal*I (two-base fill-in) sites of pHIPX4–HIS6. Subsequently, a 1·9 kb *Bam*HI (filled-in)–*Xba*I fragment containing the in-frame *PEX1*–6HIS fusion gene was ligated between the *Bam*HI (filled-in) and *Xba*I sites of the pMal–C2 vector.

For *PEX6*, the region encoding the first 559 amino acids of HpPex6p was isolated by PCR using the PEX6–ATG primer (see above) and the PEX6–HIS primer (5′ GAT GTC GAC TGT AGC TGT TGT CTC TGA TAC G 3′). The PCR fragment was digested with *Bam*HI and *Sal*I and ligated between the *Bam*HI and *Sal*I sites of pHIPX4–HIS6. Finally, the fusion gene

was isolated with *Bam*HI and *Xba*I and cloned between the *Bam*HI and *Xba*I sites of pMal–C2.

The fusion proteins were isolated either using the Protein Fusion and Purification System, as described by the supplier (New England Biolabs, Beverly, MA), or by affinity purification on a Ni-NTA column (Qiagen, Santa Clarita, CA), and were subsequently used to immunize rabbits.

### *Two-hybrid methodology*

Fusion genes between *PEX1* or *PEX6* and regions encoding the *S. cerevisiae GAL4* DNA activating (*GAL4AD*) or binding domains (*GAL4BD*) were constructed as follows. The entire *PEX1* coding region was isolated as a *Bam*HI–*Xho*I fragment from pHIPX4–PEX1 and cloned between the *Bam*HI and *Sal*I sites of pGAD424 and pGBT9 (both supplied with the MATCHMAKER system, ClonTech). Also, the entire *PEX6* coding region was isolated from pHIPX4–PEX6 as a *Not*I (blunted)–*Hpa*I fragment and cloned in *Bam*HI+*Sal*I (both blunted)-digested pGBT9. In addition, a *Pvu*II–*Eco*RV fragment from pHIPX4–PEX6 encoding HpPex6p minus the first 21 amino acids was cloned into *Bam*HI (blunted) pGAD424. Co-transformation of two-hybrid vectors into *S. cerevisiae* SFY526 and HF7c, and detection of β-galactosidase activity in transformants and cell extracts, were performed according to the instructions of the supplier of the MATCHMAKER two-hybrid system (ClonTech). HF7c transformants were also tested for their ability to grow on SD-medium without histidine.

### *Biochemical methods*

Preparation of crude extracts were performed as described by Waterham *et al.* (1994). Protoplasts were generated and lysed according to Van der Klei *et al.* (1998b). Post-nuclear supernatants were loaded onto discontinuous sucrose gradients, as described by Douma *et al.* (1985). Organellar pellets were subjected to high-salt treatment, according to Baerends *et al.* (1996). Protease protection was performed according to Van der Klei *et al.* (1998a). Co-immunoprecipitations were performed in the presence of $MgCl_2$ and ATP, according to Faber *et al.* (1998). Protein concentration determinations, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were performed using established methods.

### *Electron microscopy*

Cells were fixed and prepared for electron microscopy and immunocytochemistry, as described previously (Waterham *et al.*, 1994). Immunolabelling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against selected *H. polymorpha* peroxisomal proteins.

## RESULTS

### *Cloning of the* H. polymorpha PEX1 *and* PEX6 *genes*

*H. polymorpha per4-152* and *per5-127* are peroxisome-deficient (*pex*) mutants which were selected from a collection of mutants defective in growing on methanol ($Mut^-$). The phenotypes of the two mutants were similar in that they show defects in the import of peroxisomal matrix proteins. Immunocytochemical experiments revealed that in methanol-induced cells of both mutants only a few, small peroxisomal structures were present, which contained a minor portion of the matrix proteins alcohol oxidase (AO) (Figure 2),* catalase (CAT) and dihydroxyactone synthase (DHAS) (not shown). The bulk of these proteins were mislocated in the cytosol. Frequently, a cytosolic AO crystalloid was observed, indicative of the peroxisome-deficient phenotype of *H. polymorpha pex* mutants (Van der Klei *et al.*, 1996). The *per4-152* and *per5-127* mutants were functionally complemented using an *H. polymorpha* genomic library and restoration of the ability to grow on methanol ($Mut^+$ phenotype) as selection criterion. Sequence analysis revealed in each case a single large open reading frame (ORF) encoding a protein that was highly similar to members of the AAA protein family. The *per4-152*-complementing ORF encoded a protein of 1074 amino acids that was most similar to *P. pastoris* and *S. cerevisiae* Pex1p (43 and 33% identity, respectively) (Erdmann *et al.*, 1991; Heyman *et al.*, 1994), while the 1135 amino acid protein encoded by the *per5-127*-complementing ORF was most similar to Pex6p from *P. pastoris*, *Y. lipolytica* and *S. cerevisiae* (49, 41 and 36% identity, repectively) (Voorn-Brouwer *et al.*, 1993; Spong and Subramani, 1993; Nuttley *et al.*, 1994). From this

*Note on figures: electron micrographs are of $KMnO_4$-fixed cells unless otherwise indicated. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The marker represents 0·5 μm.

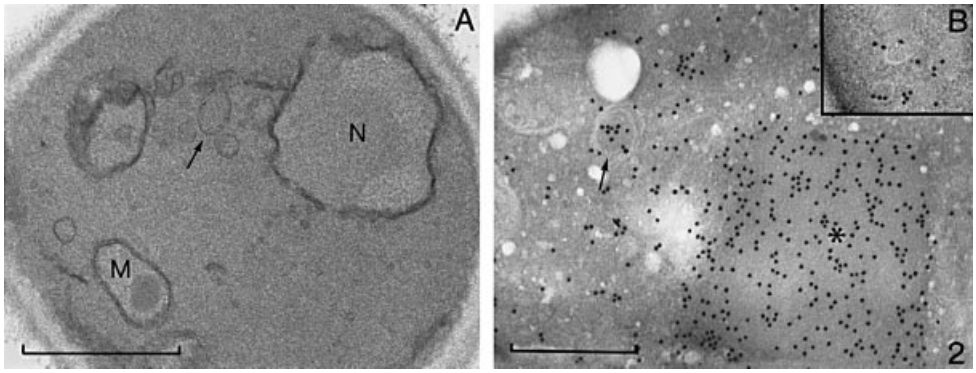



Figure 2. Morphology of the *Hansenula polymorpha per4-152* and *per5-127* mutants. (A) Morphology of a methanol-induced cell of *per4-152*, containing the few small peroxisomal remnants typical for these cells (arrow). (B) Section of a cell of *per5-127*, labelled using α-AO antibodies. Labelling is evident on the small peroxisomal remnants (arrow), the cytosolic crystalloid (*) and the cytosol. (Inset) Immunocytochemical experiment, using α-HpPex3p antiserum, showing labelling on the small peroxisomal remnants in *per4-152* cells (aldehyde, α-AO/GAR-gold, uranylacetate).

Table 1. Sequence comparison between the ATP binding motifs observed in the AAA modules in Pex1p orthologues from man (Hs), *H. polymorpha* (Hp), *P. pastoris* (Pp) and *S. cerevisiae* (Sc) and in Pex6p orthologues from man, rat (Rn), *H. polymorpha*, *P. pastoris*, *Y. lipolytica* (Yl) and *S. cerevisiae*.

| | AAA module 1 | | AAA module 2 | |
|---|---|---|---|---|
| | Walker A | Walker B | Walker A | Walker B |
| Hs-Pex1p | 599 GGKGSGKS | 662 DDLD | 881 GPPGTGKT | 940 DEFE |
| Hp-Pex1p | 504 GASGSGKT | 567 ENLD | 771 GYPGCGKT | 830 DEFD |
| Pp-Pex1p | 523 GTSGSGKS | 585 EDLD | 840 GYPGCGKT | 899 DEFD |
| Sc-Pex1p | 461 GKQGIGKT | 525 DNVE | 738 GYPGCGKT | 797 DEFD |
| | *****• | ••• | ****** | ***• |
| Hs-Pex6p | 470 GPPGCGKT | 529 TAVD | 744 GPPGTGKT | 803 DELD |
| Rn-Pex6p | 470 GPPGSGKT | 529 TALD | 742 GPPGTGKT | 801 DELD |
| Hp-Pex6p | 571 MARCVGKA | 632 RHIE | 853 GPPGTGKT | 912 DELD |
| Pp-Pex6p | 570 LSRAIGKS | 631 KHIE | 859 GPPGTGKT | 918 DELD |
| Yl-Pex6p | 477 AKRGVGKS | 536 QHLE | 760 GPPGTGKT | 819 DELD |
| Sc-Pex6p | 483 TTNNVGKA | 548 AHLD | 772 GPPGTGKT | 831 DEID |
| | **• | •• | ******** | **•* |
| Consensus | GxxGxGKT AS | DexD e | GxxGxGKT AS | DexD E |

The Walker A and B motifs of P-loop ATPases are indicated. * = Identical residues. • = Similar residues. The Walker A and B consensus motifs are also shown.

we concluded that the fragments complementing *per4-152* and *per5-127* specified HpPex1p and HpPex6p, respectively.

All Pex1p and Pex6p orthologues identified so far contain two AAA modules at the C-terminus and an approximately 500 amino acid residues-long N-terminal region that is poorly conserved. Table 1 shows a comparison of the ATP-binding motifs observed in the AAA modules of various Pex1ps and Pex6ps. As expected, the highest similarity of HpPex1p and HpPex6p to their presumed orthologues was observed in the second AAA module. Obvious organellar targeting sequences were not observed in either HpPex1p or HpPex6p. Also, putative membrane-spanning regions were absent.

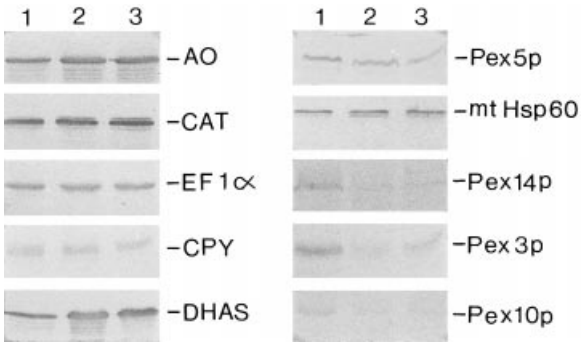



Figure 3. Protein levels in *H. polymorpha* Δ*pex1* and Δ*pex6* cells compared with WT cells. Western blots of crude extracts prepared from cells grown on methanol/glycerol mixtures were decorated with specific antisera against the proteins indicated. Lane 1, WT; lane 2, Δ*pex1*; and lane 3, Δ*pex6*. AO, alcohol oxidase: CAT, catalase; CPY, vacuolar carboxypeptidase Y; DHAS, dihydroxyacetone synthase; EF1α, cytosolic elongation factor 1-α; mt Hsp60, mitochondrial Hsp60. Equal amounts of protein were loaded per lane.

### *Characterization of* PEX1 *and* PEX6 *deletion mutants*

PEX1 and *PEX6* deletion strains (Δ*pex1* and Δ*pex6*) were constructed by replacing the regions encoding amino acids 7–504 of HpPex1p and amino acids 22–902 of HpPex6p by the *H. polymorpha URA3* gene (see Figure 1A, B). Like the original mutants, both deletion strains were unable to grow on methanol, but grew at wild-type (WT) rates on YPD or mineral media supplemented with glucose or glycerol.

To examine whether the absence of HpPex1p or HpPex6p influenced the levels of other peroxins or peroxisomal matrix proteins, Western blotting experiments were carried out using crude extracts prepared from methanol-induced cells of WT, Δ*pex1* and Δ*pex6* cells. The major peroxisomal matrix enzymes AO, DHAS and CAT, were present in Δ*pex1* and Δ*pex6* cells at levels similar to those detected in WT cells (Figure 3). Also, the PTS1 receptor HpPex5p was present at comparable amounts in extracts prepared from Δ*pex1*, Δ*pex6* and WT cells. However, the levels of the peroxisomal membrane proteins HpPex3p, HpPex10p and HpPex14p were significantly reduced in both deletion strains compared to the WT control. The levels of proteins located in the cytosol or in other cell organelles, used as additional controls, were similar in all three strains analysed (Figure 3).

The subcellular morphology of methanol-induced cells of Δ*pex1* or Δ*pex6* was highly comparable to that of the original mutants (Figure 4). Cells of Δ*pex1* or Δ*pex6* characteristically contained few small peroxisomal structures, often located in the vicinity of the nucleus. Immunocytochemical experiments revealed that these organelles were indeed peroxisomal in nature because they contained peroxisomal membrane (HpPex3p, Figure 4B; HpPex10p and HpPex14p, not shown) and matrix proteins (AO, Figure 4C). However, using antibodies against AO, the cytosol was also densely labelled, indicating that the bulk of this matrix protein was mislocated to the cytosol. Similar results were obtained when specific antibodies against other matrix enzymes were used (the PTS1 proteins DHAS and CAT and the PTS2 protein amine oxidase, data not shown). Defects resulting from deletion of either *PEX1* or *PEX6* were fully restored after reintroduction of the corresponding gene (Figure 4D; shown for *PEX6*).

Notably, the peroxisomal defect in *pex1* mutants (Δ*pex1*, *per4-152*) could not be restored by introduction of additional copies of *PEX6* under control of its own or the strong alcohol oxidase promoter ($P_{AOX}$). Also the reverse, restoration of the defect in peroxisome biogenesis in *pex6* mutants (Δ*pex6*, *per5-127*) by (over)expression of *PEX1* was not observed (data not shown).

### *HpPex1p and HpPex6p co-sediment with peroxisomal membranes*

Specific polyclonal antibodies were raised against the non-homologous N-termini of HpPex1p and HpPex6p. Western blots of crude extracts prepared from methanol-induced WT cells, and decorated with the anti-HpPex1p antibodies, revealed a faint protein band with an apparent molecular weight of approximately 125 kDa (Figure 5A). This band was absent in extracts prepared from identically-grown Δ*pex1* cells. In extracts prepared from cells overproducing HpPex1p, the intensity of this protein band was significantly enhanced, indicating that the 125 kDa band indeed represented HpPex1p. Similar results were obtained using anti-HpPex6p antibodies: a protein band with an apparent molecular weight of approximately 130 kDa was recognized in crude extracts of WT and HpPex6p overproducing cells but could not be detected in extracts of Δ*pex6* cells (Figure 5B). Also at enhanced levels of Pex1p and Pex6p, conditions to be applied in overexpression studies (see below), both antisera appeared to be fully specific (Figure 5C, D).

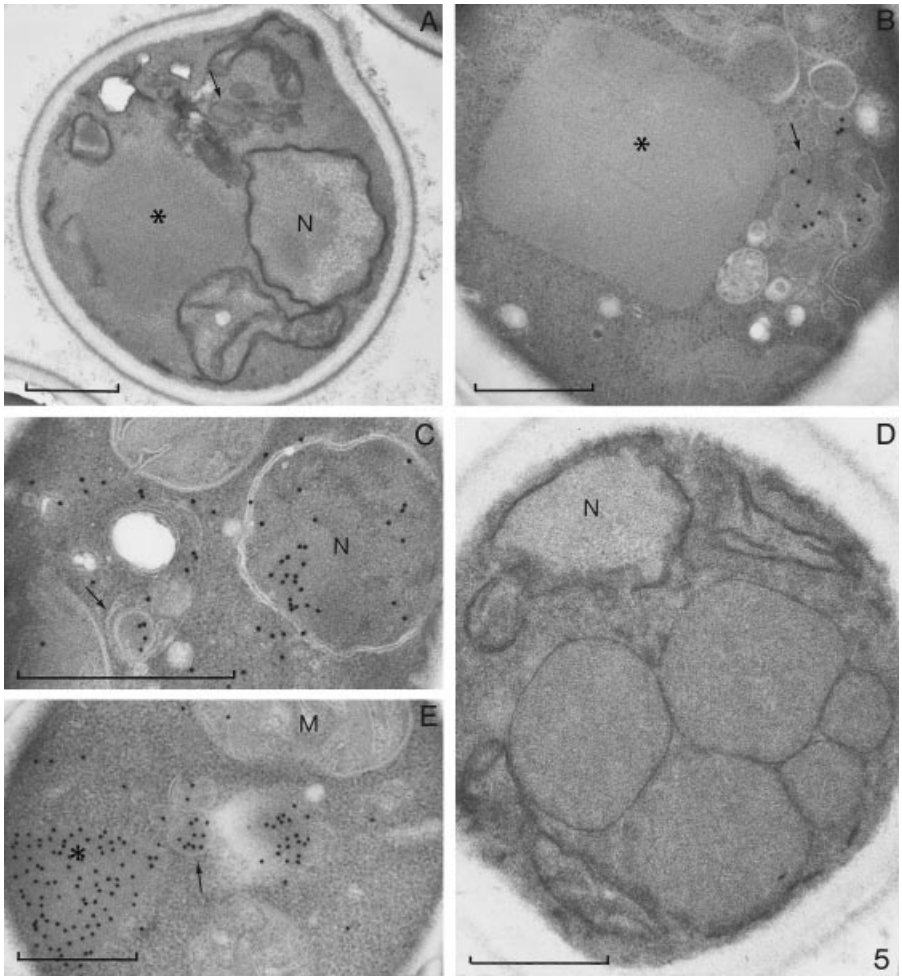



Figure 4. *PEX1* and *PEX6* deletion strains are characterized by the presence of peroxisomal ghosts. (A) The small peroxisomal remnants (ghosts, arrow) and the cytosolic AO crystalloid (*) in methanol-induced cells of Δ*pex6*. The ghosts represent the sole sites of HpPex3p (B, Δ*pex1*) and also contain AO protein (C, Δ*pex1*, arrow; E, Δ*pex6*, arrow). (D) Restoration of the normal WT phenotype of the complemented *PEX6* deletion strain (Δ*pex6*::$[P_{AOX}PEX6]^{1x}$) (B, C, E, aldehyde, indicated antisera/GAR-gold, uranylacetate).

The antisera were subsequently used to determine the subcellular location of HpPex1p and HpPex6p biochemically. Upon sucrose density centrifugation of a post-nuclear supernatant (PNS) of methanol-grown WT cells, both HpPex1p and HpPex6p co-sedimented with the peroxisomal marker proteins AO (matrix) and HpPex3p (membrane) at approximately 53% sucrose (Figure 6A, fraction 6). To investigate the possibility that HpPex1p and HpPex6p are bound to structures other than peroxisomes, which sediment at the same position in the gradient—as reported for *P. pastoris* Pex1p and Pex6p (Faber *et al.*, 1998), the distribution of HpPex1p and HpPex6p was also determined in gradients prepared from homogenates of methanol-induced Δ*pex4* (Van der Klei *et al.*, 1998a), Δ*pex8* (Waterham *et al.*, 1994) or Δ*pex14* (Komori *et al.*, 1997) cells. These strains lack intact peroxisomes but instead contain small peroxisomal ghosts due to specific defects in peroxisomal matrix protein import. As a consequence, these ghosts sediment at much lower densities, compared to intact WT peroxisomes, in a conventional sucrose gradient. In such gradients, HpPex1p and HpPex6p invariably co-sedimented with the peroxisomal membrane marker protein

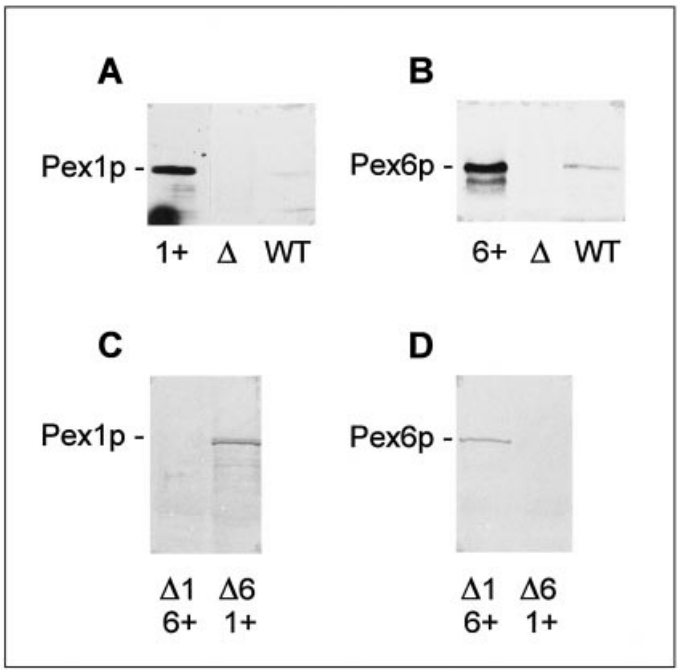



Figure 5. Specificity of the α-HpPex1p and α-HpPex6p antisera. (A–D) Western blots of crude extracts prepared from methanol-induced *H. polymorpha* cells decorated using α-HpPex1p (A and C) or α-HpPex6p (B and D) antibodies. (A) lane 1+, WT::[$P_{AOX}$*PEX1*]$^{4x}$; lane Δ, Δ*pex1*; lane WT, wild type. (B) Lane 6+, Δ*pex6*::[$P_{AOX}$*PEX6*]$^{5x}$; lane Δ, Δ*pex6*; lane WT, wild-type. (C and D) Lane Δ1/6+, Δ*pex1*::[$P_{AOX}$*PEX6*]$^{4x}$; and lane Δ6/1+, Δ*pex6*::[$P_{AOX}$*PEX1*]$^{4x}$. Equal amounts of protein were loaded per lane with the exception of lanes 1+ and 6+ (A and B), in which five-fold less protein was used.

HpPex3p (shown for Δ*pex8* in Figure 7) at densities corresponding to approximately 36% sucrose. Hence, also in ghost-containing Δ*pex* mutants, HpPex1p and HpPex6p co-fractionate with peroxisomal membranes.

To determine the subperoxisomal localization of HpPex1p and HpPex6p, a 30 000 × *g* organellar pellet fraction was subjected to high-salt extraction. The results, shown in Figure 6B, revealed that both HpPex1p and HpPex6p were fully solubilized after this treatment, whereas the membrane protein HpPex3p, used as control, remained fully pelletable. From these data we conclude that HpPex1p and HpPex6p are either peroxisomal matrix proteins or are loosely membrane-bound. To determine whether the proteins are associated with the outer surface of the peroxisome or located inside the peroxisomal matrix, we exposed purified WT peroxisomal fractions to proteinase K in the presence and absence of Triton X-100. This treatment resulted in the complete degradation of both HpPex1p and HpPex6p, independent of the solubilization of the membrane by Triton X-100 (Figure 6C). Under these conditions, the matrix protein CAT was protected. Consequently, both proteins are not peroxisomal matrix proteins but membrane-associated proteins that face the cytosol.

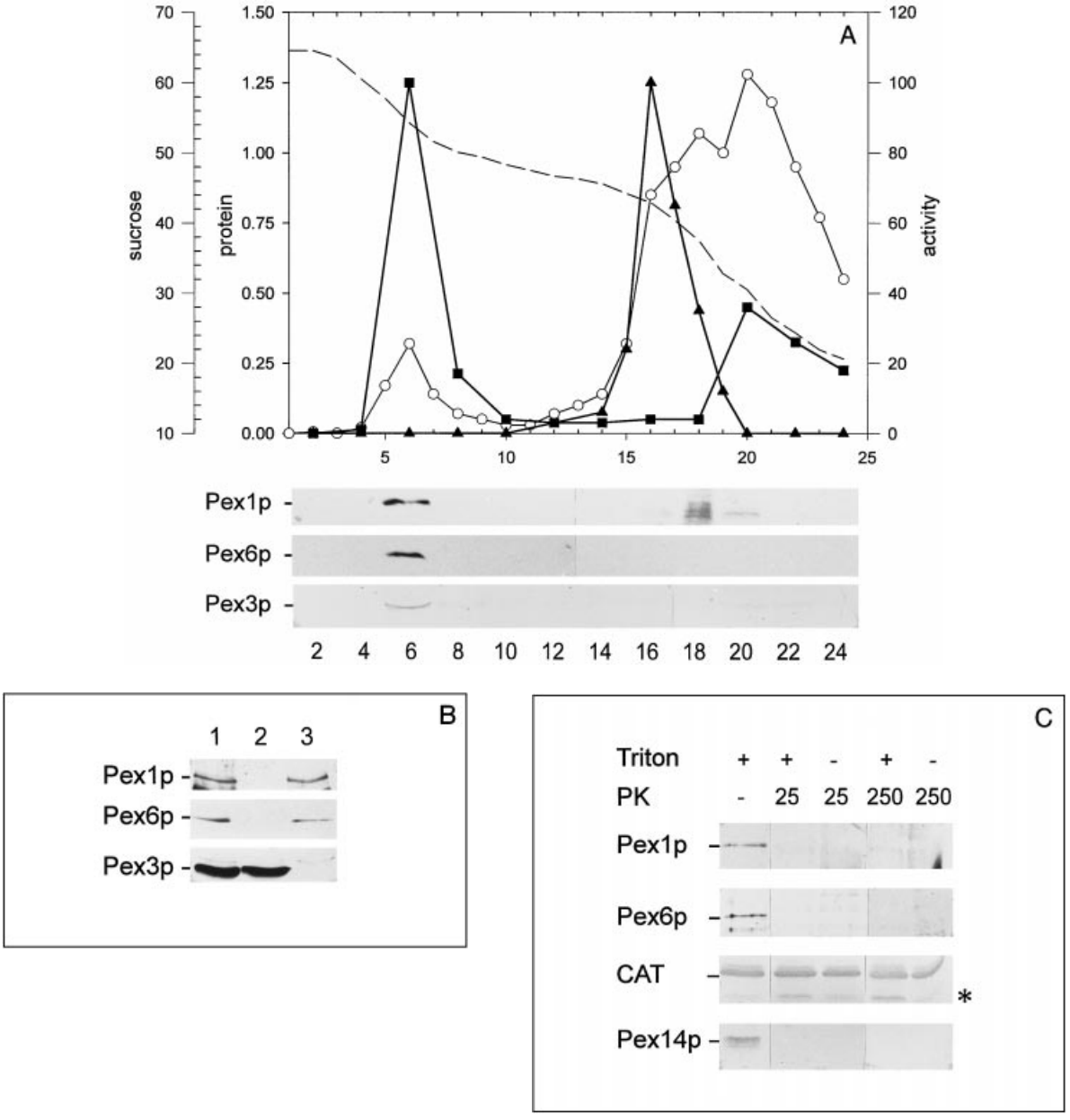
Unfortunately, immunocytochemical experiments to localize HpPex1p and HpPex6p in WT *H. polymorpha* cells were inconclusive, even in strains overproducing HpPex1p and/or HpPex6p.

### *Overexpression of* PEX6 *in* H. polymorpha *results in aberrant peroxisome assembly and a defect in matrix protein import*

In previous studies we and others have shown that overproduction of certain peroxins may strongly affect peroxisome biogenesis (see Komori *et al.*, 1997; Baerends *et al.*, 1997a,b). Analysis of these defects may give clues on the function of

these proteins. Therefore, we have studied the effect of overproduction of HpPex1p or HpPex6p on peroxisome assembly in *H. polymorpha*. Strains were constructed that contained either one or multiple copies of a cassette containing *H. polymorpha PEX1* or *PEX6* under control of the *AOX* promoter ($P_{AOX}$) integrated in their genomes. Multi-copy overexpression of *PEX1* in WT or Δ*pex1* cells did not affect growth of cells on methanol and peroxisome assembly. In methanol-grown WT::[$P_{AOX}$*PEX1*]$^{4x}$ cells, normal peroxisomes were observed which were the sole sites of AO, DHAS and CAT, as judged from immunocytochemistry (shown for AO in Figure 8A). This result was confirmed by the analysis of sucrose gradients prepared from these cells, which revealed that similar to gradients prepared from WT cells, a protein peak was present at high density in which



AO activity, HpPex3p, HpPex1p and HpPex6p co-fractionated (Figure 9A; fractions 3–8). Compared to gradients prepared from WT cells, the level of HpPex1p in the peroxisomal peak fractions had significantly increased (compare Figure 6A). In addition, the distribution of HpPex1p showed some trailing to fractions of lower density (up to fraction 12).

In contrast, overproduction of HpPex6p interfered with peroxisome assembly, which was reflected in reduced growth yields in batch cultures containing methanol as sole carbon source. A clear dose–response relationship was observed: WT or Δ*pex6* cells containing one copy of the $P_{AOX}$*PEX6* cassette grew normally on methanol-containing medium ($OD_{663}$=3·2 after 24 h). In these cells the size of peroxisomes was slightly reduced, but no matrix protein import defect was observed (data not shown). However, growth of cells containing five copies of the $P_{AOX}$*PEX6* expression cassette was significantly affected ($OD_{663}$=1·8 after 24 h). In these cells, both the number and size of peroxisomes had considerably decreased relative to those in WT control cells (Figure 8B, compare Figure 4D). Immunocytochemical experiments revealed that a significant portion of AO, DHAS and CAT protein was mislocalized to the cytosol in these cells (Figure 8D; DHAS and CAT not shown), indicating that HpPex6p overproduction gave rise to a defect in matrix protein import. Upon sucrose density centrifugation of homogenates of Δ*pex6*::$[P_{AOX}PEX6]^{5x}$ cells, only a minor AO activity peak was observed at high density (fractions 6–8, Figure 9B), whereas the bulk of the AO activity was present on the top of the gradient, which contains the soluble cytosolic proteins (fractions 21–27). The sedimentation pattern of HpPex3p had also changed, as it was now detectable in fractions 7–20. In these fractions the overproduced HpPex6p was present as well. Moreover, in the top fractions (21–27), an additional strong band of reduced apparent molecular mass was evident on Western blots decorated with anti-Pex6p antibodies. This band most likely represents a soluble degradation product of HpPex6p. HpPex1p could not be detected in sucrose gradients prepared from HpPex6p-overproducing cells. A possible explanation for this is that HpPex1p became distributed over several fractions of the gradient instead of being concentrated in only a few, which may have hampered its detection.

In order to investigate whether co-overexpression of *PEX1* could restore matrix protein import in a strain carrying multiple copies of $P_{AOX}$*PEX6*, we introduced one copy of the $P_{AOX}$*PEX1* cassette into the genome of WT::$[P_{AOX}PEX6]^{5x}$. The resulting strain grew normally on methanol (final $OD_{663}$, 3·3) and the cells contained peroxisomes of normal size. Immunocytochemical experiments indicated that import of AO protein was restored (Figure 8C), although not completely. The protein and AO activity profiles in sucrose gradients prepared from these cells revealed the re-appearance of the peroxisomal peak at high density (Figure 9C, fractions 5–7). These fractions contained a minor portion of the total amount of HpPex1p and HpPex6p produced in these cells. The bulk of HpPex1p was found in fractions 19–23. HpPex6p was found predominantly in fractions 19–27, with trailing to fraction

Figure 6. Biochemical localization of HpPex1p and HpPex6p. (A) Sucrose gradient, prepared from a post-nuclear supernatant of methanol-grown *H. polymorpha* WT cells. Sucrose (% w/v, – – –), protein concentrations (mg/ml, ○—○), the distribution of the activities of the peroxisomal marker alcohol oxidase (■), and the mitochondrial marker cytochrome *c* oxidase (▲) are indicated. Enzymatic activities are expressed as percentages of the activities of the peak fractions, which were set to 100%. Western blots show the distribution of HpPex1p, HpPex6p and the peroxisomal membrane protein HpPex3p in the even fractions of the gradient (HpPex1p and HpPex6p detected using chemiluminescence techniques; HpPex3p detected using alkaline phosphatase). All three proteins co-sediment with high density peroxisomes in fraction 6. Equal portions of each fraction were loaded per lane. (B) A 30 000 × *g* organellar pellet prepared from methanol-grown WT *H. polymorpha* cells (lane 1) was subjected to high-salt treatment by incubation in 0·5 M NaCl for 30 min at 0°C. The membrane-bound proteins (lane 2) and soluble proteins (lane 3) were separated by centrifugation for 30 min at 100 000 × *g*. HpPex1p and HpPex6p are fully soluble after this treatment; HpPex3p is not solubilized at all (all proteins detected using chemiluminescence techniques). Equal portions of the pellet and soluble fractions were loaded per lane. (C) Protease protection assay using purified peroxisomes obtained from a sucrose gradient from an *H. polymorpha* WT post-nuclear supernatant. Equal amounts of protein were incubated with proteinase K (PK, μg/ml) in the absence (−) or presence (+) of 0·1% Triton X-100 for 30 min at 0°C. Western blots were decorated with α-HpPex1p, α-HpPex6p, α-HpPex14p and α-catalase (CAT) antibodies. The controls show that the peroxisomal matrix protein CAT is partially digested in the presence of both proteinase K and Triton X-100, as illustrated by the increase of the amount of a smaller protein band (*). CAT is fully protected in the absence of Triton X-100. Degradation of HpPex14p, which is known to reside at the cytosolic face of the peroxisomal membrane, is dependent on proteinase K, but independent of Triton X-100. Degradation of HpPex1p and HpPex6p is also independent of Triton X-100.

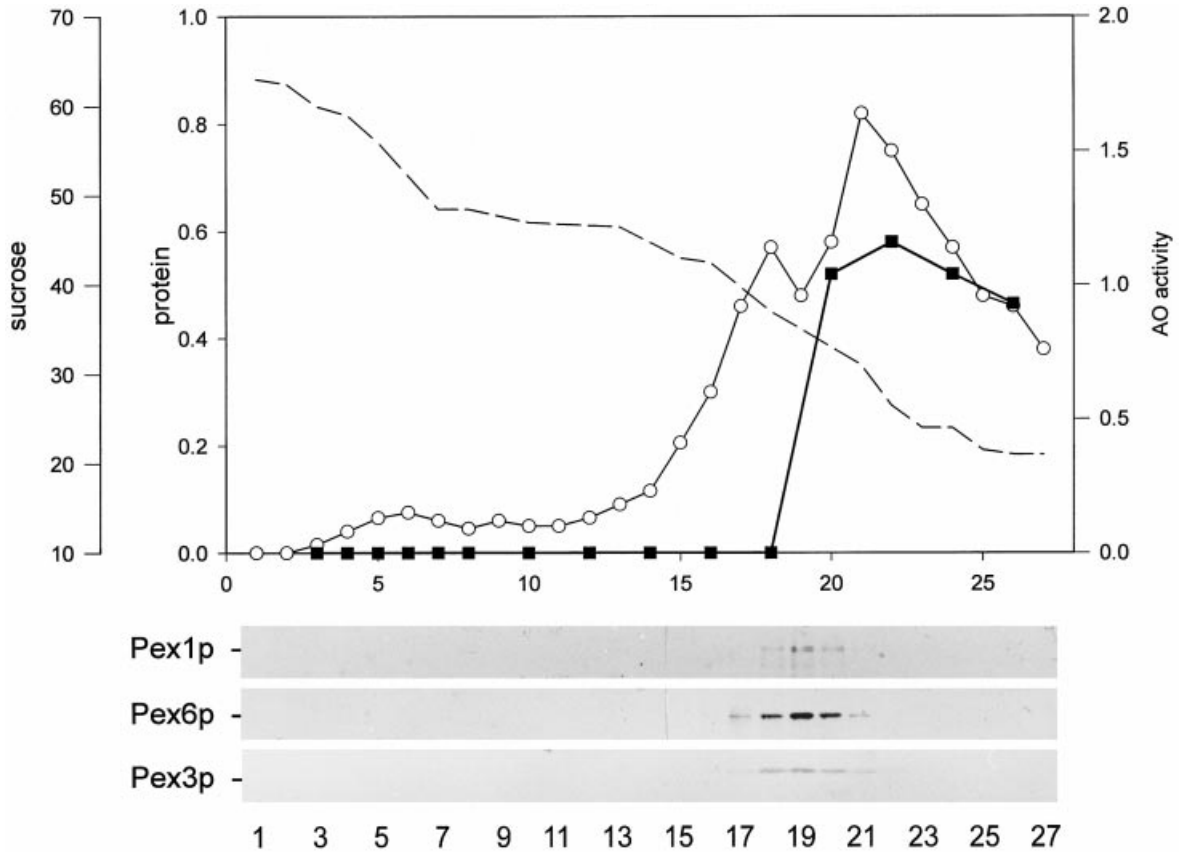



Figure 7. Localization of HpPex1p and HpPex6p in Δ*pex* mutants. Sucrose gradient prepared from a post-nuclear supernatant of methanol-induced *H. polymorpha* Δ*pex8* cells. The graph shows the sucrose (% w/v, – – –) and protein concentration patterns (mg/ml, ○—○) and the distribution of the activity of the peroxisomal marker alcohol oxidase (in U/ml, ■). Western blots show the distribution of HpPex1p, HpPex6p and HpPex3p. The three proteins co-sediment in fractions 18–20 at approximately 36% sucrose. Equal portions of each fraction were loaded.

12. The putative HpPex6p degradation band in the upper fractions of the gradient was significantly reduced when HpPex1p was co-overexpressed (compare Figure 8B). The peroxisomal membrane protein HpPex3p co-fractionated with peroxisomes (fraction 5–7), but a significant portion of this protein was also found in lower-density fractions (13–21). Therefore, these fractions may contain small, immature peroxisomes or membrane vesicles.

### *HpPex1p and HpPex6p interact* in vivo

The overproduction studies with HpPex1p and HpPex6p described above suggested that these proteins functionally interact. To study this in more detail, two-hybrid analyses were performed (Fields and Song, 1989). Fusion constructs were prepared by inserting most of the coding regions of *PEX1* and *PEX6* in plasmids encoding either the activation or the DNA-binding domain of *S. cerevisiae* Gal4p. In-frame fusion genes were co-expressed in *S. cerevisiae* strains containing the *E. coli lacZ* gene and/or the *S. cerevisiae HIS3* gene under the control of Gal4p-regulated promoters. Physical interaction between HpPex1p and HpPex6p was expected to result in β-galactosidase production and/or histidine-prototrophy. The results, shown in Table 2 and Figure 10A, indicated that a strong activation of the *lacZ* and/or *HIS3* transcription occurred only in double transformants carrying either *GAL4BD–PEX1* and *GAL4AD–PEX6* or *GAL4BD–PEX6* and *GAL4AD–PEX1*. No activation was observed in

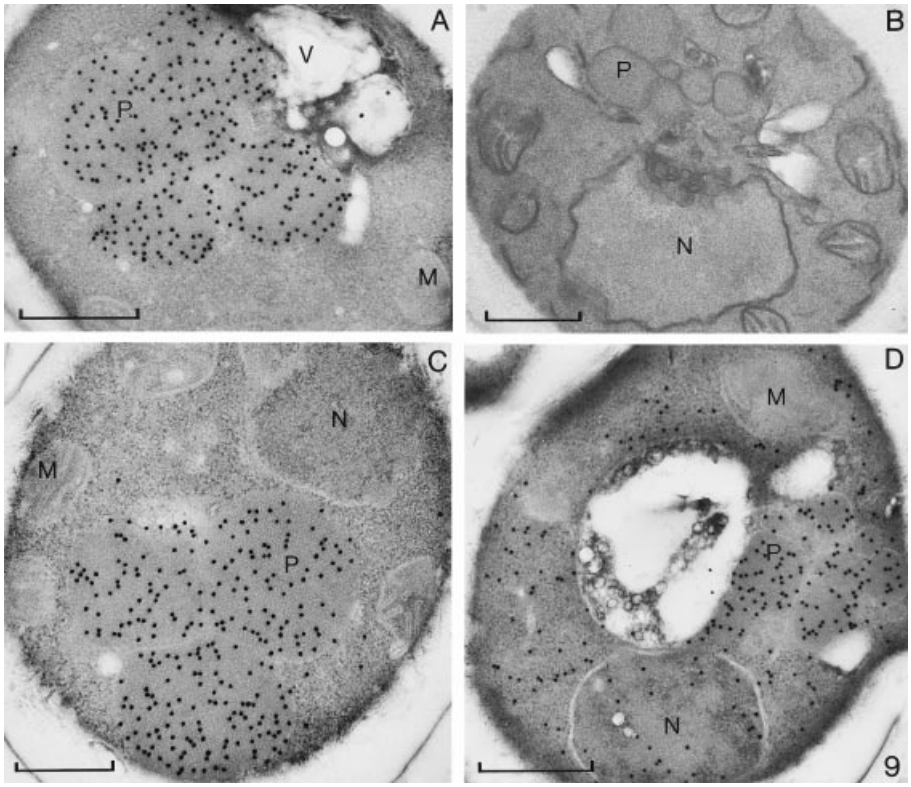



Figure 8. Overexpression of *PEX1* and *PEX6* in *H. polymorpha*. (A) Methanol-grown WT::[$P_{AOX}$*PEX1*]$^{4x}$ cell, showing normal peroxisomes with AO protein confined to peroxisomes (α-AO). (B) Overall cell morphology of Δ*pex6*::[$P_{AOX}$*PEX6*]$^{5x}$ showing the relatively small peroxisomes present in these cells. The organelles contain AO protein, which is also located in the cytosol, and in the nucleus (cf. D, α-AO). (C) In WT::[$P_{AOX}$*PEX6*]$^{5x}$::[$P_{AOX}$*PEX1*]$^{1x}$ cells co-overexpressing *PEX6* and *PEX1*, peroxisome morphology is normal again and AO protein is almost completely inside peroxisomes (α-AO). (D) Control to (C) representing an identical-grown WT::[$P_{AOX}$*PEX6*]$^{5x}$ cell in which the vector pHI1 was integrated at the *URA3* locus (α-AO) (aldehyde, indicated antisera/GAR-gold, uranylacetate).

double transformants carrying exclusively *PEX1* or *PEX6* in frame with both *GAL4AD* and *GAL4BD*.

Further evidence for a physical interaction between HpPex1p and HpPex6p *in vivo* came from co-immunoprecipitation experiments. Using the α-HpPex1p antibodies HpPex6p was co-immunoprecipitated with HpPex1p when extracts of methanol-grown WT cells were used (Figure 10B). The amount of precipitated HpPex1p and HpPex6p was significantly enhanced when cells were used which overproduced both HpPex1p and HpPex6p. In a control experiment, using an extract prepared from a strain lacking HpPex1p but overproducing HpPex6p, no HpPex6p was precipitated by the HpPex1p antibodies, indicating that HpPex6p indeed had co-precipitated with HpPex1p in experiments using WT or the double overproducing strain. As expected, only HpPex1p was precipitated by the HpPex1p antiserum when an extract was used from a strain overproducing HpPex1p but lacking HpPex6p.

## DISCUSSION

We have cloned and characterized the *PEX1* and *PEX6* genes of the yeast *H. polymorpha*. Both genes encode proteins belonging to the AAA family of ATPases and are involved in peroxisome biogenesis. HpPex1p and HpPex6p are loosely associated with the cytosolic face of the peroxisomal membrane and functionally and

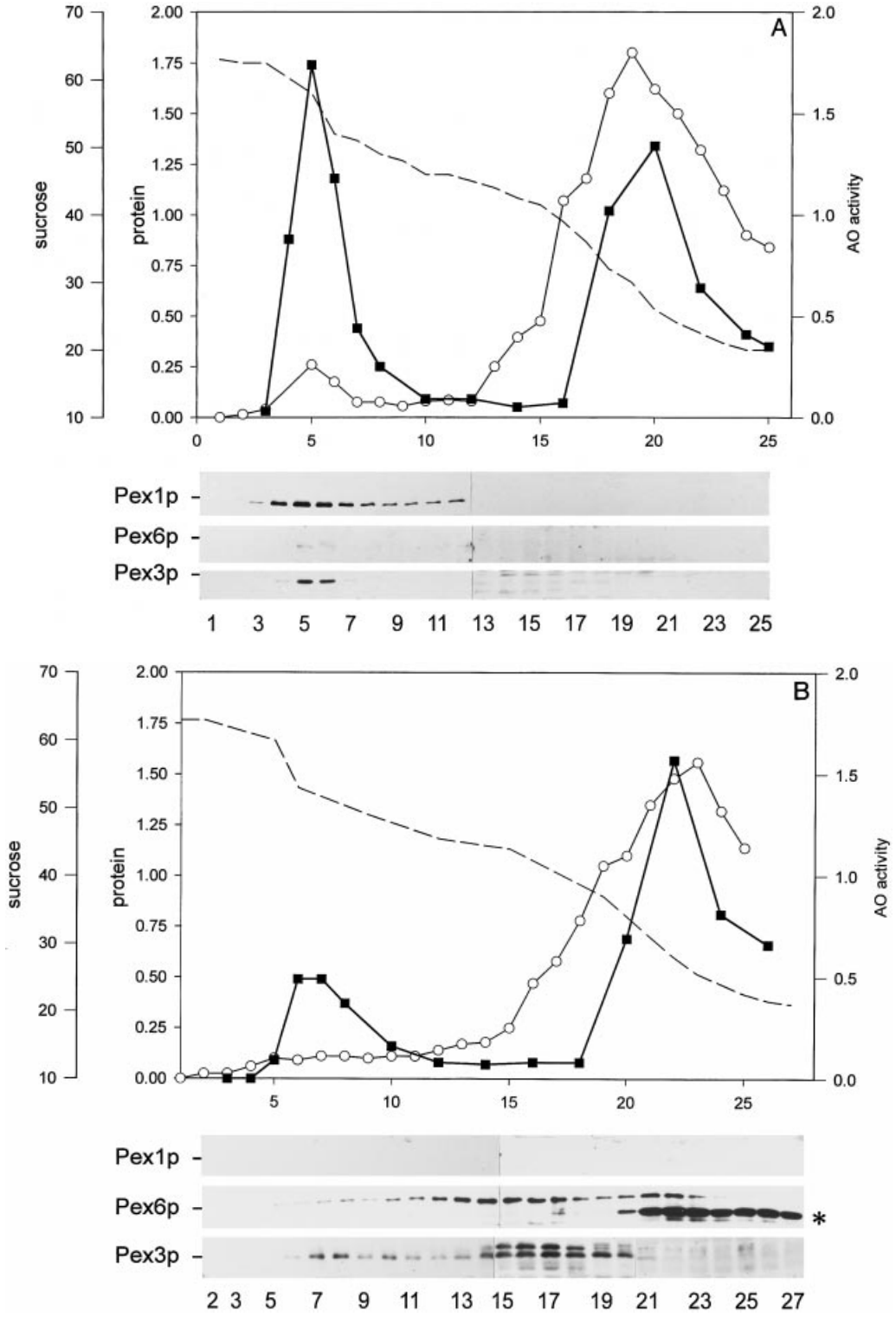



Figure 9. (A and B).

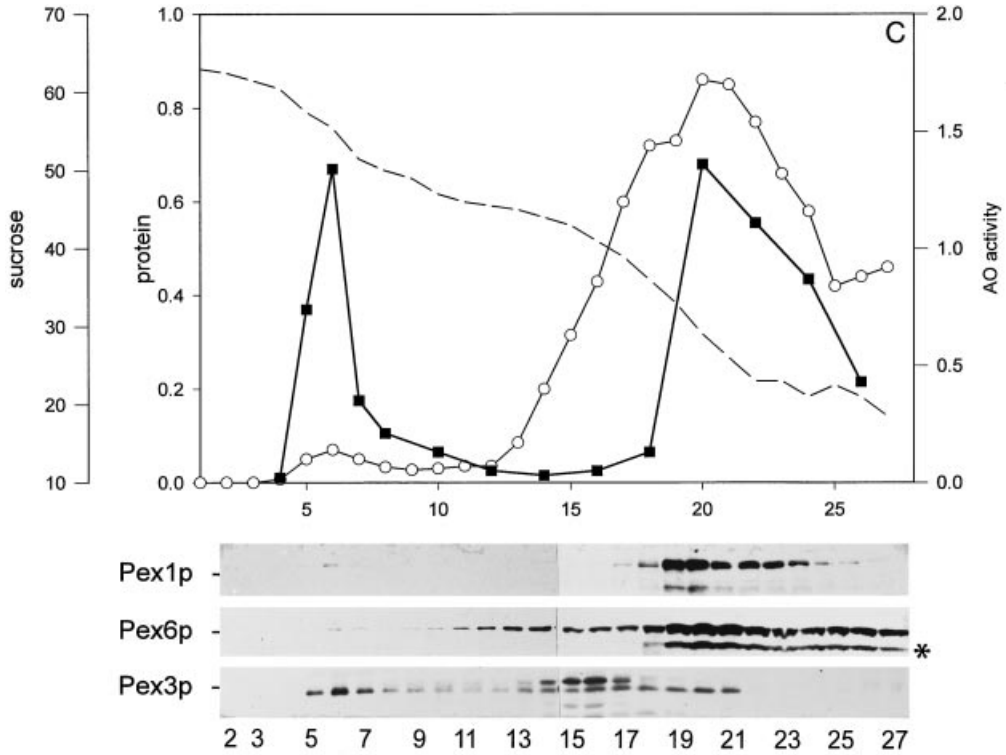



Figure 9. (C).

Figure 9. Cell fractionation of methanol-grown Δ*pex1*::[$P_{AOX}$*PEX1*]$^{4x}$ (A), Δ*pex6*::[$P_{AOX}$*PEX6*]$^{5x}$ (B) and WT::[$P_{AOX}$*PEX6*]$^{5x}$::[$P_{AOX}$*PEX1*]$^{1x}$ (C) cells. Sucrose density gradients were prepared from post-nuclear supernatants. The graphs show the sucrose (% w/v, – – –) and protein concentration patterns (mg/ml, ○—○) and the distribution of the activity of the peroxisomal marker alcohol oxidase (U/ml, ■). Western blots show the distribution of HpPex1p, HpPex6p and HpPex3p. Equal portions of each fraction were loaded per lane. In (B) and (C), (*) indicates a protein band that is specifically recognized by the α-HpPex6p antiserum and presumably represents a degradation product of HpPex6p. A cross-reacting protein band of slightly higher molecular weight is sometimes recognized by the α-HpPex3p antibodies in fractions containing the mitochondria (fractions 14–18 in B and C).

physically interact. In addition, *PEX6* overexpression results in peroxisome dysfunction, a defect that was rescued by concomitant co-overexpression of *PEX1*.

Like Pex1ps and Pex6ps from other organisms, the C-terminal halves of HpPex1p and HpPex6p contain two AAA modules. The various Pex1ps and Pex6ps differ with respect to the degree of conservation in the AAA modules: in Pex1ps the Walker A and B motifs are readily recognized in both modules, but in Pex6ps only the second module is conserved, whereas the first shows little resemblance to the AAA module (Table 1). The ATP-binding site in the highly conserved, second AAA module has been shown to be essential for the function of both Pex1p and Pex6p (Krause *et al.*, 1994; Tsukamoto *et al.*, 1995; Yahraus *et al.*, 1996). Various AAA proteins implicated in vesicular transport or membrane fusion processes (e.g. *S. cerevisiae* Sec18p and Cdc48p and their orthologues) also contain two AAA modules in the C-terminal half of the proteins (see Confalonieri and Duguet, 1995).

The morphology of methanol-induced cells of *H. polymorpha* Δ*pex1* or Δ*pex6* strains was comparable. These cells harboured few small peroxisomal remnants (ghosts), which were characterized by the presence of peroxisomal membrane proteins and low amounts of the major matrix enzymes, which predominantly resided in the cytosol (Figure 4). In a previous communication, we already demonstrated that the

Table 2. Two-hybrid interactions between HpPex1p and HpPex6p.

| | Gal4-BD fused to: | Gal4-AD fused to: | β-Galactosidase activity (U) |
|---|---|---|---|
| + | Murine p53 | SV40 large T-antigen | 169·4 |
| – | — | — | 0·15 |
| 1 | — | Pex1p | 0·09 |
| 2 | — | Pex6p (22-1135) | 0·06 |
| 3 | Pex1p | — | 0·06 |
| 4 | Pex6p | — | 0·15 |
| 5 | Pex1p | Pex1p | 0·10 |
| 6 | Pex6p | Pex6p (22-1135) | 0·11 |
| 7 | Pex6p | Pex1p | 48·6 |
| 8 | Pex1p | Pex6p (22-1135) | 19·1 |

The amount of β-galactosidase activities in *S. cerevisiae* SFY526 double-transformants producing the indicated combinations of Gal4p-peroxin fusion proteins in given; 1 unit of activity is defined as the amount of β-galactosidase which hydrolyses 1 μmol ONPG to *O*-nitrophenol and D-galactose per minute.

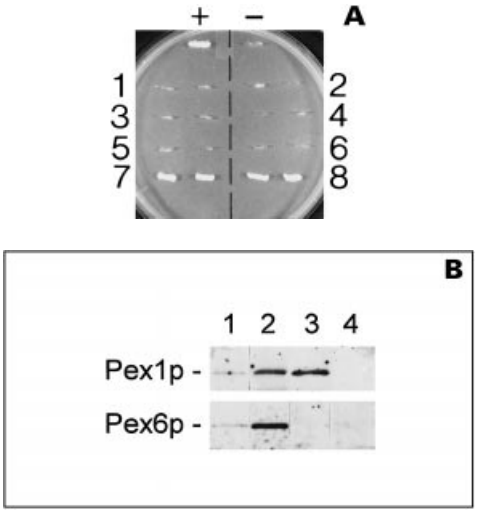



Figure 10. *H. polymorpha* HpPex1p and HpPex6p interact with each other. (A) Analyses of growth of *S. cerevisiae* HF7c double-transformants producing fusion proteins identical to those in Table 2 on SD plates without histidine. In both cases, only double transformants producing both Gal4p–HpPex1p and Gal4p–HpPex6p fusion proteins produce a positive result. The positive control, consisting of plasmids pVA3 and pTD1, was supplied with the MATCHMAKER system. (B) Co-immunoprecipitation using anti-HpPex1p antiserum and lysates from *H. polymorpha* WT (lane 1), WT::$[P_{AOX}PEX6]^{5x}$::$[P_{AOX}PEX1]^{1x}$ (lane 2), Δ*pex6*::$[P_{AOX}PEX1]^{4x}$ (lane 3) and Δ*pex1*::$[P_{AOX}PEX6]^{4x}$ (lane 4). Western blots were decorated with α-HpPex1p or α-HpPex6p antibodies, as indicated.

peroxisomal ghosts in Δ*pex1* and Δ*pex6* cells display peroxisomal characteristics because of their ability to proliferate upon overproduction of HpPex10p (Veenhuis *et al.*, 1996). In addition, like intact peroxisomes in WT cells, these ghosts were shown to be susceptible to glucose-induced proteolytic degradation. Peroxisomal ghosts have also been observed in *P. pastoris* Δ*pex1* and Δ*pex6* cells (Spong and Subramani, 1993; Heyman *et al.*, 1994) and in *S. cerevisiae* Δ*pex1* cells (Purdue and Lazarow, 1995).

Our data demonstrate that both HpPex1p and HpPex6p are associated with membranes and face the cytosol (Figure 6). Furthermore, after sucrose density gradient centrifugation, both proteins invariably migrate to the same position as HpPex3p-containing peroxisomal membranes, independent of their density—at 53% sucrose in case of WT peroxisomes (Figure 6A) and at 36% sucrose in case of small peroxisomes/ghosts from three separate Δ*pex* strains (Figure 7). This suggests that both proteins are associated with the peroxisomal membrane. Recently, Faber *et al.* (1998) showed that in the yeast *P. pastoris*, PpPex1p and PpPex6p are located on separate membranous structures that were distinct from peroxisomes. Formally, we can not exclude the possibility that in WT *H. polymorpha* also, HpPex1p and HpPex6p are located on structures other than peroxisomal membranes. Their sedimentation behaviour in sucrose density gradients would then be explained by assuming that these structures are firmly associated with the peroxisomal membrane. However, we consider this possibility less likely, because morphological support is lacking. Localization of HpPex1p and HpPex6p on structures other than peroxisomal membranes was observed in HpPex1p and HpPex6p co-overproducing strains. However, under these conditions significant portions of both proteins still co-fractionated with HpPex3p. The relationship between these membranous structures and those observed in WT *P. pastoris* remains to be established.

Co-fractionation of HpPex1p and HpPex6p in sucrose density gradients is in line with the results from the two-hybrid studies and co-immunoprecipitation experiments, which show that HpPex1p and HpPex6p physically interact. This has also been demonstrated for Pex1p and Pex6p from *P. pastoris* and man (Faber *et al.*, 1998; Tamura *et al.*, 1998b; Geisbrecht *et al.*,

1998). Previous genetic studies on *H. polymorpha PEX1* and *PEX6* already suggested that these genes are functionally linked (Titorenko *et al.*, 1993), a finding which is confirmed by our overexpression studies.

In mammals, the studies on the localization of Pex1p and Pex6p gave contradictory results. In rat liver cells, Pex6p was shown to be a peroxisomal protein, tightly bound to the peroxisomal membrane (Tsukamoto *et al.*, 1995). In man, Pex1p and Pex6p were reported to be cytosolic proteins (Yahraus *et al.*, 1996; Tamura *et al.*, 1998b). However, in these studies both human proteins were epitope-tagged and overproduced, conditions that may have resulted in an artificial location.

To gain insight in the possible function of HpPex1p and HpPex6p, we performed overexpression studies of *PEX1* and *PEX6* in *H. polymorpha*. Overproduction of HpPex1p did not affect peroxisome biogenesis/function. Notably, all overproduced HpPex1p was membrane-bound, and predominantly co-fractionated with high density peroxisomes (Figure 9A). Overproduction of HpPex6p clearly affected peroxisome function, and resulted in a matrix protein import defect. Morphologically, the overproduction of HpPex6p in *H. polymorpha* did not result in the excessive membrane proliferation that was observed after overproduction of the peroxisomal membrane proteins HpPex3p or HpPex14p (Komori *et al.*, 1997; Baerends *et al.*, 1997a,b). Under *PEX6*-overexpressing conditions, HpPex6p was mainly present on membranes of low density and only a minor portion co-sedimented with the peroxisomal membrane marker protein HpPex3p (Figure 9B). Possible explanations as to why HpPex6p overproduction affects peroxisome function include: (a) HpPex6p directly interferes with peroxisome assembly and/or matrix protein import, e.g. by disturbing the stoichiometry of the proteins in the putative translocation complex; (b) the excess amounts of mislocalized HpPex6p prevent HpPex1p reaching its site of activity. This second possibility is supported by two important observations. First, in cells overproducing HpPex6p, HpPex1p could not be detected at its normal peroxisomal location, although it was synthesized at WT levels. Second, co-overexpression of *PEX1* in cells overproducing HpPex6p rescued the peroxisomal import defect. In these cells only a minor amount of HpPex1p and HpPex6p co-sedimented with peroxisomes at high density (Figure 9C). Apparently, this small amount of HpPex1p is sufficient for proper peroxisome functioning. However, in this strain the majority of HpPex1p and HpPex6p co-fractionated at low density. Whether these low-density structures are actual intermediates in the process of peroxisome biogenesis, as has been suggested for *P. pastoris* (Faber *et al.*, 1998), remains to be established. Clearly, a well-balanced HpPex1p–HpPex6p interaction at the peroxisomal membrane is essential for normal peroxisome biogenesis/matrix protein import in *H. polymorpha*. This observation is in line with the recent finding of Geisbrecht *et al.* (1998), that disturbances in the interaction between Pex1p and Pex6p in man are the main cause of peroxisomal disorders.

The key question that remains is how the two AAA proteins function in peroxisome biogenesis. In all organisms studied so far, malfunction of either Pex1p or Pex6p leads to severe matrix protein import defects. Basically, these import defects may be explained by: (a) a deficiency in the matrix protein import machinery; or (b) a defect in the development of the peroxisomal membrane (e.g. a block in phospholipid transfer). Our present data do not allow us to distinguish which of these two possibilities is correct. It has been suggested that the deficiency in matrix protein import in *P. pastoris* Δ*pex6* cells and human fibroblasts, obtained from patients suffering from peroxisomal biogenesis disorders in complementation groups 1 (Pex1$^-$) and 4 (Pex6$^-$), is related to insufficient amounts of the PTS1 receptor, Pex5p (Yahraus *et al.*, 1996; Reuber *et al.*, 1997). However, in *H. polymorpha*, deletion of either *PEX1* or *PEX6* did not significantly affect the levels of HpPex5p. Furthermore, we recently found that overproduction of HpPex5p in Δ*pex1* and Δ*pex6* cells does not rescue the Pex$^-$ phenotype in these cells (F. A. Salomons, in preparation). From this we conclude that in *H. polymorpha* maintenance of the stability/function of the PTS1 receptor HpPex5p is not the primary function of Pex1p or Pex6p.

An observation that could be in line with the second possibility is that in *H. polymorpha* Δ*pex1* and Δ*pex6* cells, the levels of the membrane-bound peroxins HpPex3p, HpPex10p and HpPex14p were reduced (Figure 3). In cells of other *H. polymorpha* Δ*pex* strains, these reduced amounts of membrane proteins were not observed (see Baerends *et al.*, 1996; Van der Klei *et al.*, 1998a). This could imply that HpPex1p and HpPex6p are important for the transport/insertion or stability of certain peroxisomal membrane proteins involved in peroxisome

biogenesis. The defect in matrix protein import in Δ*pex1* and Δ*pex6* cells could then be an indirect effect, caused by too-low levels of membrane-bound peroxins essential for matrix protein import (e.g. HpPex14p). Evidence is now accumulating that certain peroxisomal membrane proteins involved in peroxisome biogenesis may reach their target organelle via the ER by vesicle trafficking and fusion processes (Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998). If we consider the possibility that HpPex1p and HpPex6p might play a role in these processes, this would explain the reduced levels of peroxisomal membrane proteins in Δ*pex1* and Δ*pex6* cells. In such a scenario, the availability of membrane-lipid components and thus peroxisomal growth would also be affected in Δ*pex1* and Δ*pex6* cells.

In conclusion, our data suggest that HpPex1p and HpPex6p constitute a protein complex associated with peroxisomal membranes. In addition, the presence of sufficient HpPex1p at the peroxisomal membrane seems to be a prerequisite for normal peroxisome function. Previously, classical genetic studies have suggested a complex set of interactions between HpPex1p, HpPex6p and other peroxins (Titorenko *et al.*, 1993). Clearly, the identification of additional components that interact with HpPex1p and HpPex6p is required to gain a better understanding of the function of these AAA proteins in peroxisome biogenesis.

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